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## Evaluation of a novel tuberculosis complex-specific 34 kDa protein in the serological diagnosis of tuberculosis

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**ABSTRACT:** Tuberculosis (TB) serological testing with antigen complexes, although very sensitive, is not always as specific due to reactive serum antibodies in patients with inactive TB or nontuberculous infections. Since the use of recombinant *M. tuberculosis* proteins may enhance specificity, this study was designed to evaluate a novel 34 kDa tuberculosis complex-specific protein as a component of an antigen panel of recombinant proteins.

Seventy patients with active TB (41 positive and 29 negative for acid-fast bacilli (AFB) in sputum) were evaluated, in comparison with 30 tuberculin purified protein derivative skin test positive (PPD+) and 30 PPD- normals, 20 subjects with inactive TB and 20 PPD+ subjects with nontuberculous pneumonia as controls. Serum antibody levels were quantified using enzyme linked immunosorbent assay (ELISA) tests with MS2-34, a fusion protein comprising the NH<sub>2</sub>-terminal 16 kDa of the 34 kDa protein, a recombinant 38 kDa protein (p38), and PPD.

Using MS2-34 and p38 as an antigen panel in active TB patients yielded higher sensitivity and negative predictive value (sensitivity 86%; negative predictive value 91%) than using PPD (sensitivity 66%; negative predictive value 81%). Importantly, the MS2-34+p38 panel yielded a higher sensitivity (83%) than PPD (66%) in the subset of AFB- active TB patients.

Thus, this novel protein increases sensitivity and specificity of serological testing for TB when used in panels of recombinant proteins.

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Since the worldwide resurgence of tuberculosis (TB), the development of rapid diagnostic tests is becoming increasingly important. Up to 50% of *M. tuberculosis* culture confirmed TB patients present with negative acidfast bacilli (AFB) smears [1–4], and receive empirical treatment until culture results are obtained. Rapid culture methods, molecular assay, and serological assays may all play important roles in securing a rapid diagnosis, directing decision making on isolation and treatment of patients, and on screening of contacts.

In recent years, serological tests for TB have been developed using *M. tuberculosis* extracts, such as purified protein derivative of the tuberculin (PPD), the antigen complex A60 or purified antigens and recombinant proteins [5, 6]. However, *M. tuberculosis* expresses broadly cross-reactive antigens that may be recognized by non-specific antibody reactions in: 1) healthy individuals [7]; 2) *M. tuberculosis*-exposed contacts [7]; 3) persons with inactive tuberculosis [5, 6, 8]; and 4) patients with mycobacteria other than tuberculosis (MOTT) infections [9]. Immunodominant *M. tuberculosis* proteins, which perform with higher sensitivity and specificity in diagnostic serology, have been isolated in several laboratories by

screening deoxyribonucleic acid (DNA) libraries with immune sera or with immune T-lymphocytes [10, 11].

This study was designed to evaluate diagnostic sensitivity and specificity of a novel tuberculosis complex-specific recombinant protein of 34 kDa [12, 13]. An enzyme linked immunosorbent assay (ELISA) test using a fusion protein (MS2-34) comprising the 16 kDa NH<sub>2</sub>-terminal fragment of the 34 kDa protein and an 11 kDa fragment of the MS-2 polymerase fusion partner indicated elevated anti-34 kDa antibody levels in the sera of patients with TB. By combining this test with an ELISA using the *M. tuberculosis*-specific recombinant 38 kDa protein (p38) [14, 15], it was possible to diagnose TB in AFB smear-negative patients with high sensitivity and specificity.

### Methods

#### *Production of the recombinant MS2-34 fusion protein*

Partial expression of the 34 kDa antigen gene [16] was obtained as a 27 kDa fusion protein [17]. Briefly, a

BamHI-EcoRI mycobacterial DNA fragment was inserted in pBluescript (Stratagene, Cambridge, UK), recovered by restriction enzyme digestion as a BamHI-HindIII fragment, and reinserted into pEX34 c (Promega Corp., Madison, WI, USA) to obtain a fusion protein (hereafter referred to as MS2-34) comprising the NH<sub>2</sub>-terminal 142 amino acids of the 34 kDa protein and an 11 kDa fragment of the MS2 polymerase. The recombinant plasmid pSBc was used to transform *E. coli* K12  $\Delta$ H  $\Delta$ trp cells [18]. Bacterial cultures (400 mL of Luria-Bertani broth supplemented with 30  $\mu$ g·mL<sup>-1</sup> ampicillin) were induced to synthesize the fusion protein by temperature shifting (30 to 42°C). Cells were collected after 150 min (10,000  $\times$  g for 15 min), resuspended in 3.2 mL of Tris-sucrose (50 mM Tris, 25% sucrose, pH 8), incubated at 37°C for 30 min (0.8 mL of 5 mg·mL<sup>-1</sup> lysozyme and 0.8 mL of 0.5 M ethylenediamine tetra-acetic acid (EDTA)), lysed with Triton-X 100 (1% Triton 100, 50 mM Tris, 63 mM EDTA; 15 min at 0°C, 30 min at 37°C), sonicated and pelleted (15,000  $\times$  g for 10 min). The pellet was washed in 1 M urea (37°C for 30 min) and redissolved in the same buffer before purification by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution. The fusion protein concentration and purity were assessed by spectrophotometry (Lambda 5, Perkin-Elmer & Co., GmbH, Überfingn, Germany), by Western blotting with the 34 kDa proteinspecific monoclonal antibody H61.3 [12], and by analytical SDS-PAGE and silver staining (fig. 1).

#### Anti-MS2-34 serum levels

Immunoglobulin M and G (IgM and IgG) antibody levels against the 34 kDa protein were quantified by ELISA using the MS2-34 fusion protein (27 kDa) with

background subtraction of the MS-2 phage polymerase fusion partner (MS2, 11 kDa). Briefly, MS2-34 (200 ng·well<sup>-1</sup>) or an equimolar quantity of MS2 (81 ng·well<sup>-1</sup>) were adsorbed on different wells of the microtitre plate for 1 h and saturated with 3% bovine serum albumin (BSA) (Sigma, St. Louis MO, USA) in phosphate buffer saline (PBS). To quantify antibody levels, the binding of duplicate serum samples (1:50 dilution, which was established as the optimal dilution in preliminary experiments) was revealed using biotin-conjugate anti-human IgM or IgG antibodies (Sigma, St. Louis, MO, USA) and extravidin-peroxidase (Sigma) with o-phenylenediamine (Sigma) and H<sub>2</sub>O<sub>2</sub> by spectrophotometry (490 nm, ETI-System Fast Reader, Sorin Biomedica, Saluggia, Italy). Optical density (OD) values were normalized using a pool of four control sera (control), following the equation: (OD sample<sub>MS2-34</sub>/OD control<sub>MS2-34</sub>) - (OD sample<sub>MS2</sub>/OD control<sub>MS2</sub>). Tests with an intraduplicate error >20% were discarded and repeated.

#### Anti-p38 serum levels

IgM and IgG antibody levels against the *M. tuberculosis*-specific 38 kDa protein were quantified by ELISA technique using recombinant p38 (2  $\mu$ g·well<sup>-1</sup>). The binding of serum antibodies (1:100 dilution, which was established as the optimal dilution in preliminary experiments) was revealed using peroxidase conjugated anti-human IgM or IgG antibodies (Sigma, St. Louis, MO, USA).

#### Anti-PPD serum levels

IgM and IgG antibody levels against PPD were quantified by ELISA technique using PPD (Statens Seruminstitut, Copenhagen, DK; 40  $\mu$ g·well<sup>-1</sup>) as described previously

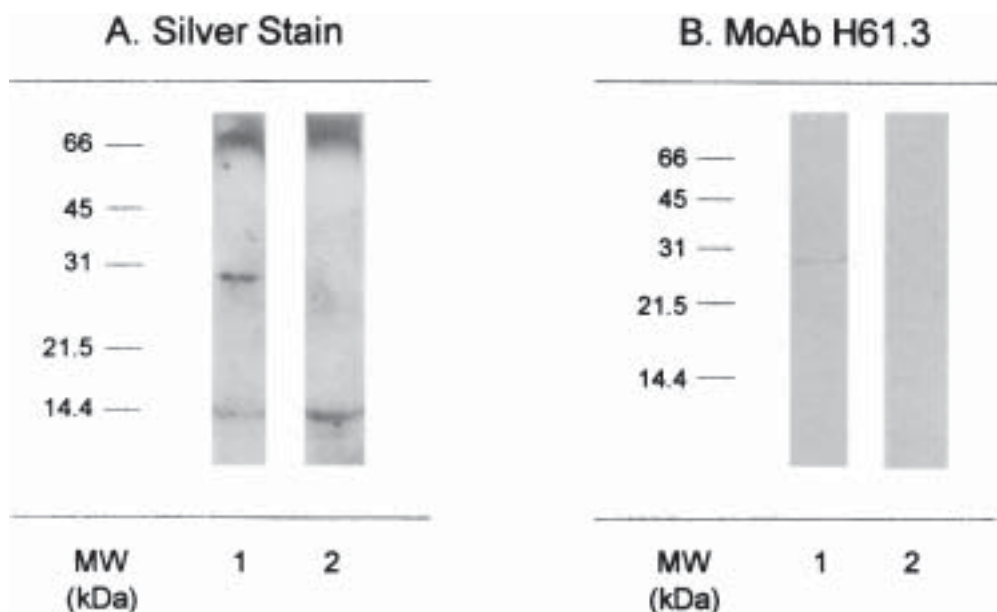


Fig. 1. – A) Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining; and B) Western blot with the monoclonal antibody H61.3 of the MS2-34 27 kDa fusion protein containing the NH<sub>2</sub>-terminal 98 amino acid of the 34 kDa tuberculosis complex protein. In each panel are shown the MS2-34 protein (lane 1) and the MS2 polymerase 11 kDa fusion partner (lane 2). Molecular weight markers are shown to the left.

[19]. Serum samples were used at the 1:500 dilution for IgM and 1:1,000 for IgG test, which were established as the optimal dilution in preliminary experiments.

PPD skin testing was performed using 5 units of the tuberculin purified protein derivative (PPD) (Sclavo, Siena, Italy; or Parke-Davis, Morris Plains, NJ, USA). An induration greater than 10 mm was scored as positive [20].

### Study populations

The study populations included PPD-negative and PPD-positive healthy subjects, PPD-positive bacterial pneumonia patients and inactive TB patients. The healthy control population included 30 PPD-positive non-bacille Calmette Guérin (BCG)-vaccinated subjects (mean age  $18 \pm 3$  yrs, 11 males and 19 females, all Caucasians); and 30 PPD-negative subjects (mean age  $17 \pm 4$  yrs, 13 males and 17 females, all Caucasians) without a history of TB.

A group of 20 PPD-positive individuals affected by nontuberculous bacterial pneumonia (mean age  $38 \pm 11$  yrs, 12 males and 8 females) was also evaluated, since the discrimination of bacterial pneumonia from TB by means of immunological tests in individuals from moderate TB prevalence areas may be confused by nonspecific recall reactions. All subjects had negative *M. tuberculosis* sputum cultures.

The nonactive TB population included 20 individuals with chemotherapy-cured pulmonary TB (mean age  $42 \pm 13$  yrs, 10 males and 10 females, all Caucasians), who were evaluated after 12–24 months from the discontinuation of specific treatment. All had negative chest radiographs.

The active TB-affected population included 70 individuals with postprimary untreated pulmonary TB (mean age  $41 \pm 17$  yrs, 41 males and 29 females, 56 Caucasians, 1 African, 2 Asians, 11 Afro-Americans), who were evaluated at the time of admission before the initiation of antituberculous therapy. Average length of time with symptoms before admission, that was evaluated on 34 subjects, was  $3.3 \pm 2.9$  months. All had *M. tuberculosis* culture-positive sputum.

All patients were evaluated after obtaining informed consent under protocols approved by the Ethics Committee of the University of Modena (Italy) and the Georgetown University (Washington DC, USA).

### Statistical evaluation

Data are presented as means  $\pm$  standard deviation of the mean. ELISA tests were scored positive when individual readings were greater than three standard deviations above the control average (expected specificity 99.6%). Comparisons between group antibody levels were analysed using the Student's t-test. Comparisons between the frequencies of positive and negative responders were analysed using the Chi-squared test with Bonferroni correction. Sensitivity, specificity and predictive value were evaluated as described by DANIEL and DEBANNE [5].

## Results

### *Anti-MS2-34 levels in patients with active tuberculosis*

Patients with active untreated TB had markedly higher anti-MS2-34 IgM and IgG antibody levels (IgM,  $0.50 \pm 0.31$  and IgG,  $0.38 \pm 0.26$  ELISA units) compared to PPD-positive normal controls (IgM,  $0.12 \pm 0.05$  and IgG,  $0.11 \pm 0.04$  ELISA units), or PPD-negative normal controls (IgM,  $0.07 \pm 0.03$  and IgG,  $0.08 \pm 0.04$  ELISA units) ( $p < 0.01$ ) (fig. 2), and to pneumonia-affected individuals (IgM,  $0.11 \pm 0.04$  and IgG,  $0.12 \pm 0.03$  ELISA units;  $p < 0.01$ ) (fig. 2). Anti-MS2-34 IgM and IgG antibody levels were also significantly higher in active TB patients compared to individuals with inactive, chemotherapy-cured TB (IgM,  $0.06 \pm 0.04$  and IgG,  $0.09 \pm 0.11$  ELISA units;  $p < 0.01$ ) (fig. 2).

### *Sensitivity and specificity of the MS2-34 ELISA test*

The IgM ELISA with MS2-34 (cut-off value 0.29 ELISA units) was as sensitive and specific, since the cut-off value was above the normal range (table 1), as the ELISA with p38 (cut-off value 1.10 ELISA units) and PPD (cut-off value 0.94 ELISA units). In comparison to all groups (PPD-negative and PPD-positive controls, patients with bacterial pneumonia, and patients with inactive TB) the anti-MS2-34 IgM specificity was 100% (99% confidence interval (99% CI) 95–100%) *i.e.* similar to that of the anti-p38 IgM test (100% (99% CI 95–100)) and the anti-PPD IgM test (100% (99% CI 95–100)). Moreover, since the IgM test MS2-34 (cut-off value 0.28 ELISA units) was negative in almost all of inactive TB controls (fig. 2b), the specificity of the IgG test with this protein was as high as the IgM test. On the contrary, the p38 (cut-off value 0.76 ELISA units; fig. 2d) and the PPD (cut-off value 0.67 ELISA units; fig. 2f) IgG test specificities were lower than the IgM tests (MS2-34 95% (99% CI 71–99); p38 40% (99% CI 16–68);  $p < 0.01$  compared to MS2-34; PPD 25% (99% CI 7–53);  $p < 0.001$  compared to MS2-34).

### *Sensitivity of the MS2-34 and p38 ELISA tests combined*

When the two MS2-34 and p38 IgM ELISA tests were evaluated as a test panel, combining the results obtained for each antigen in separate assay plates, their rate of positive tests (86% (33% to MS2-34 only; 21.5% to p38 only; 31.5% to both antigens)) was significantly higher than for each antigen alone and for the PPD ( $p < 0.02$ , all comparisons) (table 1). They also had a higher negative predictive value than PPD (MS2-34 and p38 combined negative predictive value 91% (99% CI 82–96); PPD 81% (99% CI 71–88);  $p < 0.03$ ). As expected from the lower p38 test specificity (see fig. 2), whilst the use of the IgM test combination significantly improved the test performance, the use of the two IgG tests combined did yield the same sensitivity as the IgM test (81% (24% to

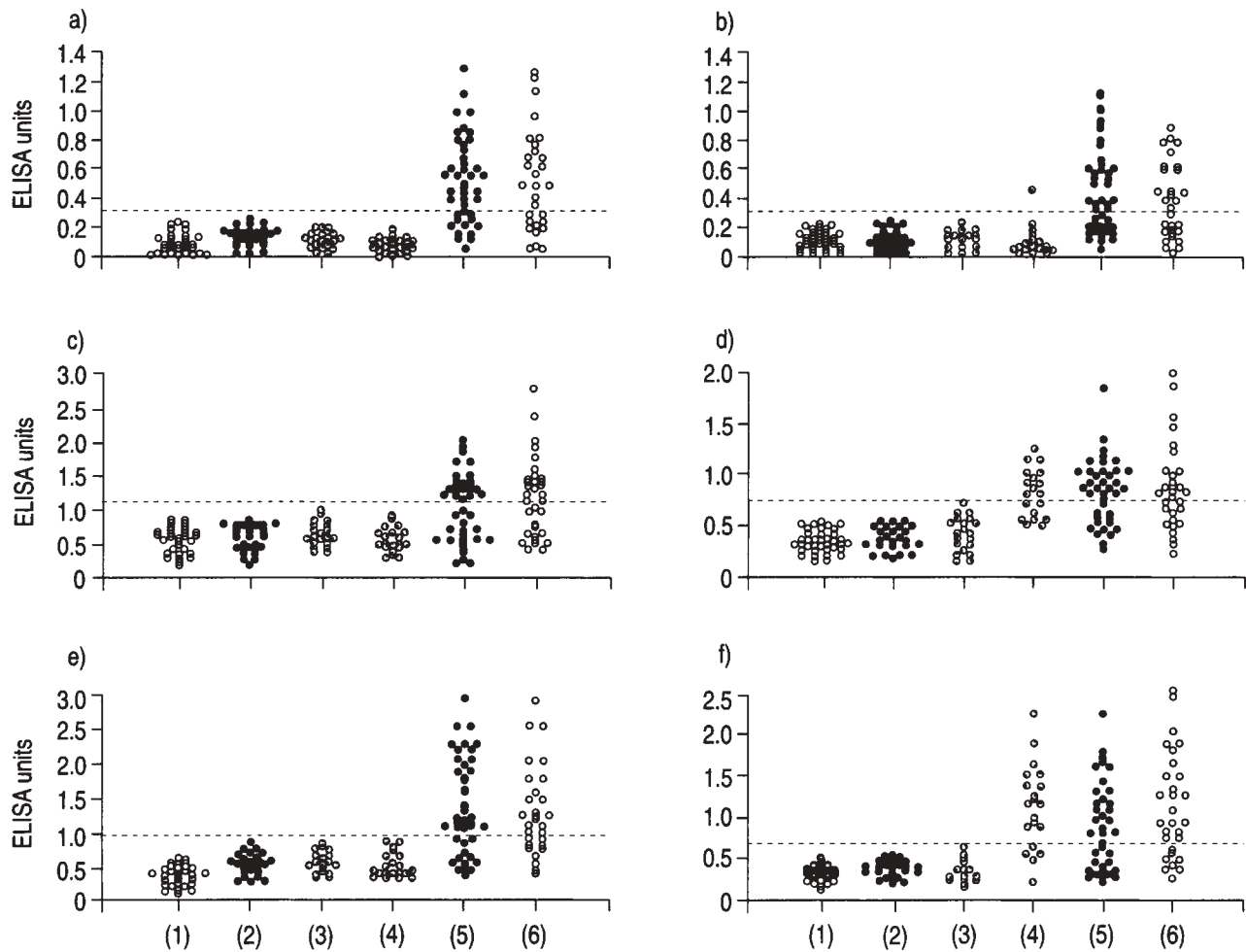


Fig. 2. – Quantification of anti-p34 serum antibodies by ELISA with the MS2-34 recombinant fusion protein. On the ordinate (as normalized ELISA units) are shown the levels of: a) IgM against MS2-34; b) IgG against MS2-34; c) IgM against p38; d) IgG against p38; e) IgM against PPD; f) IgG against PPD. Cut-off values for each ELISA test (3 SD above the mean value of controls) are shown as a dotted line. Study groups are shown on the abscissa. They were: PPD negative normal controls (1); PPD positive normal controls (2); patients with bacterial pneumonia (3); individuals with inactive pulmonary tuberculosis (4); patients with AFB-positive active tuberculosis (5); and patients with AFB-negative active tuberculosis (6). ELISA: enzyme-linked immunosorbent assay; IgM and IgG: immunoglobulin M and G; p38: a recombinant 38 kDa protein; PPD: purified protein derivative skin test; AFB: acid-fast bacilli. For further abbreviations see legend to figure 1.

Table 1. – Sensitivity of IgM ELISA testing with MS2-34, p38 and PPD for the diagnosis of active tuberculosis

	Controls			Tuberculosis	
	PPD- (n=30) %	PPD+ (n=30) %	Pneumonia (n=20) %	Inactive (n=20) %	Active (n=70) %
IgM-MS2-34	0	0	0	0	67*
IgM-p38	0	0	0	0	54*
IgM-PPD	0	0	0	0	66*
IgM-(MS2-34+p38)	0	0	0	0	86*§

IgM: immunoglobulin M; ELISA: enzyme-linked immunosorbent assay; MS2-34: a fusion protein comprising the NH<sub>2</sub>-terminal 98 amino acid of the 34 kDa tuberculosis complex protein; p38: a recombinant 38 kDa protein; PPD- and PPD+: purified protein derivative skin test negative and positive, respectively. \*:  $p < 0.05$  vs controls (all comparisons); §:  $p < 0.01$  compared to IgM-MS2-34, IgM-p38 and IgM-PPD.

MS2-34 only; 24% to p38 only; 33% to both antigens)) but had lower specificity.

#### *Evaluation of ELISA tests in active TB smear-negative vs smear-positive and PPD-negative vs PPD-positive patients*

The MS2-34 ELISA test was positive in similar proportions of AFB-negative and AFB-positive patients ( $p > 0.6$ ) (table 2). Furthermore, when the MS2-34 and p38 IgM ELISA tests were evaluated in combination, a higher proportion of AFB smear-negative and smear-positive active TB patients could be identified than with MS2-34, p38 and PPD alone (table 2).

The IgM MS2-34 ELISA test was positive in 75% of PPD-negative subjects ( $p > 0.2$ , compared to p38 and PPD), and in 62% of PPD-positive TB patients ( $p > 0.4$ , compared to p38 and PPD). The IgG MS2-34 ELISA



Table 2. – Sensitivity of IgM ELISA tests with MS2-34, p38, PPD and the combination of MS2-34 and p38 in the diagnosis of tuberculosis in AFB-negative and AFB-positive patients with active tuberculosis

	AFB+ (n=41) %	AFB- (n=29) %
IgM-MS2-34	71	62
IgM-p38	54	55
IgM-PPD	66	66
IgM-(MS2-34+p38)	88*§	83*§

AFB- and AFB+: acid-fast bacilli smear negative and positive, respectively. For further abbreviations see legend to table 1.

\*:  $p > 0.40$  compared to AFB smear-negatives; §:  $p < 0.05$  compared to PPD, MS2-34 alone and p38 alone.

test was positive in 50% of PPD-negative subjects ( $p > 0.3$ , compared to p38 and PPD) and in 57% of PPD-positive TB patients ( $p > 0.15$ , compared to p38 and PPD). In the eight AFB-negative active TB patients with a negative skin test, six were MS2-34 positive and seven (87%) were MS2-34 and/or p38 positive. Out of 19 AFB-negative/PPD-positives, 13 were MS2-34 positive and 16 (84%) MS2-34 and/or p38 positive ( $p > 0.4$ , PPD-negative vs PPD-positive).

## Discussion

Since 1898, when a serological assay for the diagnosis of TB was developed that showed 57% sensitivity and 89% specificity [21], tests with *M. tuberculosis* extracts, sonicates and filtrates (including antigens such as PPD and A60) have been described that showed sensitivities between 49 and 95%, with specificities between 84 and 100% [5]. A number of *M. tuberculosis* purified antigens, such as antigen 5, Lipo Arabino Mannan (LAM), Sulpho Lipid-IV (SL-IV) and proteins of 10, 19, 24 and 30 kDa and several recombinant proteins including heat shock protein 70 (HSP70), p38, p32, and p14, have also been successfully employed [7, 22–27].

This study shows that the fusion protein MS2-34, as obtained from the tuberculosis complex-specific 34 kDa protein coding gene, performs in diagnostic serology with high sensitivity and specificity. Furthermore, in the context of serological studies with other purified proteins in population groups of different geographical background [28], the comparison of anti-MS2-34 antibody levels in patients of Caucasian or Africo-American descent (data not shown), albeit preliminary, suggests that recognition of this antigen is not limited by genetic and racial factors.

The major advantage of using highly purified immunodominant reagents is that their use in multi-antigen tests or in panels of tests may greatly improve upon diagnostic sensitivity in comparison to antigen mixtures such as PPD or A60. In this regard, BOTHAMLEY *et al.* [29] have found that the combined use of tests with antigens, such as

p38, p19, p14, and LAM, and with monoclonal antibodies against them may considerably improve diagnostic sensitivity (up to 72%) compared to the single tests with LAM (32%) and with p19 (58%). VERBON *et al.* [26] have obtained similar results with antigens of 10, 16, 24, 30, 38 and 70 kDa and the monoclonal antibody TB72 (specific for the p38 protein [15]). They have shown that test combinations, such as with the 10, 16, 24 kDa antigens and the antibody TB72, could improve the diagnostic sensitivity of each test. For example, the 10 kDa antigen test showed 29% sensitivity, compared to 70% of the combined tests.

Our observations with the use of both the MS2-34 and p38 recombinant proteins support these findings. The combined MS2-34 and p38 assays' sensitivity and specificity were very good even when specificity was controlled against groups of PPD-positive healthy individuals, *i.e.* subjects naturally exposed to *M. tuberculosis*. Furthermore, the test distinguished PPD-positive patients with bacterial pneumonia presenting with a clinical picture raising the suspicion of TB. The observation that MS2-34 is specific for active TB, and it is not recognized by IgG antibodies from patients with cured or inactive disease is quite interesting, since discrimination of active from inactive patients requires many immunological markers [30]. However, the sensitivity of the MS2-34/p38 IgM test is as high as that of the MS2-34/p38 IgG test. Thus, the use of the IgG test would not improve sensitivity, whilst it may reduce specificity, since p38 is recognized by IgG serum antibodies of patients with inactive TB (this study and [31]). At the present time, we cannot explain why the IgG recognizing MS2-34 decline more rapidly than antibodies recognizing other proteins. In the context that the 34 kDa protein is the gene product of a mobile element [16], its expression may be expected to be lower or more transient than that of constitutive proteins [32]. It is, thus, possible that it may become rapidly unavailable to the immune system with the reduction of bacterial load.

With regard to the use of ELISA tests for antibodies of different isotypes, it is worth mentioning the observation of TURNER *et al.* [25], who found that the use of IgG and IgA ELISA tests with the 32 kDa antigen did improve the sensitivity of each test by 25 and 50%, respectively. In this study, however, no significant increase in the sensitivity of IgM tests was observed when IgM and IgG results were combined for either MS2-34 alone or both MS2-34 and p38.

Serological tests might play a major role in the setting of the diagnosis of AFB-negative patients with suspect TB, since in these subjects culture results may not be available until 15–40 days after clinical presentation. The data show that with the MS2-34 and p38 IgM ELISA tests, TB can be diagnosed with 83% sensitivity and greater than 95% specificity in AFB-negative patients. Furthermore, IgM tests were found to be positive in 87% of AFB-negative patients who also had a negative PPD skin test, indicating a role for serology in immunological diagnosis of TB.

With regard to the diagnosis of AFB-negative patients, BOTHAMLEY *et al.* [29] found evident differences in

the responses to p38 in AFB-negative compared to AFB-positive patients. The present study, like the study by TURNER *et al.* [25] with p32, WILKINS and IVANYI [33] with the anti-p38 TB72 antibody, and VERBON *et al.* [26] with anti-p38 TB72 antibody, the 10, 16, 24, 30, 38 and 70 kDa proteins showed similar sensitivity in AFB-negative and AFB-positive patients. These comparisons, though, are merely speculative if differences in each laboratories' proficiency with AFB smear techniques are not taken into account [1–4].

Rapid molecular tests, such as the polymerase chain reaction (PCR), are available for the diagnosis of TB in AFB-negative patients [34, 35]. However, the application of these tests is still limited to a small number of laboratories. ELISA tests, although not as sensitive as the molecular tests, are rapid, relatively inexpensive and suitable for routine use. The use of multiple-antigen ELISA tests with purified or recombinant proteins might greatly enhance the power of serological diagnosis of TB for those patients who may otherwise receive delayed treatment.

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